Lipophilic derivatives of 5-fluoro-2'-deoxyuridine as liposomal anticancer agents

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CHAPTER 8

TOXICITY OF LIPOSOMAL 3'-5'-O-DIPALMITOYL-5-FLUORO-2'-DEOXYURIDINE IN MICE

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ABSTRACT

Toxicities of 5-fluoro-2'-deoxyuridine (FUdR, 600 μmol/kg) and liposomal FUdR-dipalmitate (2 μmol/kg) to mouse bone marrow, spleen, liver and ileum were compared after treatment for 6 consecutive days. Toxicity to the hemopoietic system was more severe for FUdR than for liposomal FUdR-dipalmitate and included a decrease in progenitor and precursor cells of the erythroid and granuloid/macrophage lineage in bone marrow and spleen. Body weight and liver weight were reduced by 20% and 30% respectively and the number of mitotic cell divisions in the liver was significantly diminished for both drugs. Toxicity to the ileum was more severe for liposomal FUdR-dipalmitate than for FUdR and was manifested by granulocyte infiltration, the presence of cell debris, loss of columnar epithelial cells, and enlarged nuclei with prominant nucleoli in these cells.
Thus, by prolonging the presence of FUdR in vivo by using liposomal FUdR-dipalmitate the major toxic effects shift from bone marrow to the gastro-intestinal tract.

INTRODUCTION

The therapeutic dose of the antineoplastic agent 5-fluoro-2'-deoxyuridine (FUdR) can be reduced considerably when the drug is applied as its lipophilic prodrug 3',5'-O-dipalmitoyl-5-fluoro-2'-deoxyuridine (FUdR-dipalmitate) incorporated in liposomes (1-4). Whereas FUdR can be injected in mice repeatedly at doses up to 400 - 600 μmol/kg before major toxicity symptoms appear, the maximum tolerated dose of FUdR-dipalmitate is almost two orders of magnitude lower (2 - 10 μmol/kg). An increase in antitumor activity of liposome-prodrug formulations could not be achieved without the simultaneous induction of severe toxicity symptoms in the tumor-bearing host, resulting even in an actual decrease in therapeutic index.

The remarkable difference in tolerated dose between the liposomal prodrug and the free drug is assumed to be due to a sustained exposure effect. When FUdR is injected as a free drug, its plasma concentration declines rapidly, due to extensive catabolism, initially to 5-fluorouracil (5FU) and subsequently to inactive catabolites (5,6). When FUdR is administered as liposome-incorporated FUdR-dipalmitate however, the drug is directed mainly to the cells of the mononuclear phagocyte system (MPS) in liver and spleen (4,7,8). These cells have been shown to function as a drug-depot from which FUdR is slowly released after intralysosomal digestion of the liposomes and hydrolysis of the ester bonds in the prodrug (9, chapter 3 and 4). This implies that the exposure time of tissues (both tumor and actively dividing normal tissues) to FUdR in vivo is prolonged when the drug is given as a liposomal prodrug formulation. Because the administration of FUdR-

dipalmitate led to a reduction in the therapeutic index of FUdR, actively dividing, normal tissue appears to be particularly sensitive to the prolonged exposure to FUdR.

When FUdR is administered as an intravenous bolus injection in humans, bone marrow depression represents the dose-limiting toxicity (10, 11). However, the toxicity spectrum is shifted towards the gastro-intestinal tract when the drug is administered as an continuous, intravenous infusion (12,13) and to the liver upon intra-arterial hepatic infusion (12-15). Thus, organ toxicity depends on the route of administration and on whether the drug is given as a bolus injection or as an infusion.

In this study we examined the involvement of different organs and tissues in the occurrence of host toxicity in mice after repeated administration of liposome-associated FUdR-dipalmitate. We therefore compared the toxic effects of free drug (600 μmol/kg) and liposome-prodrug (2 μmol/kg) on bone marrow and intestine (ileum), tissues known to be involved in FUdR toxicity. Additionally, we evaluated liver and spleen, the main target organs of liposomes, with respect to possible toxic effects of FUdR and liposomal FUdR-dipalmitate. The toxicological study was done histologically for liver, intestine and spleen. Furthermore, hemopoietic progenitor cells of the spleen and the bone marrow were quantified after growth in culture.

MATERIALS AND METHODS

Chemicals

5-Fluoro-2'-deoxyuridine (Floxuridine, FUdR) was generously supplied by Hoffmann-La Roche (Basel, Switzerland). Distearoylphosphatidylcholine (DSPC) and dipalmitoylphosphatidylglycerol (DPPG) were obtained from Avanti Polar lipids, Inc. (Birmingham, AL, USA). Cholesterol (CHOL) was obtained from Sigma (St Louis, MO). FUdR-dipalmitate was synthesized as described before (3).

Preparation of liposomes

Phospholipids, CHOL and FUdR-dipalmitate were kept in chloroform/methanol (4:1) at - 20°C under nitrogen. For the preparation of liposomes aliquots of stock solutions of lipids and drug (ratio DSPC/DPPG/CHOL/FUdR-dipalmitate 10:1:10:2.1) were mixed and dried under nitrogen. The lipids were dissolved in cyclohexane, frozen and lyophilized. The lyophilized lipids were hydrated with HN buffer (10 mM Hepes, 135 mM NaCl, pH 7.4) by thorough vortexing. The resulting multilamellar vesicles (MLV's) were extruded through 0.4-μm polycarbonate membranes (Nuclepore, Pleasanton, CA) using an extruder (Lipex Biomembranes Inc., Vancouver, Canada). Phospholipid phosphorus was assessed with molybdate reagent after perchloric acid destruction (16). The content of liposomal FUdR-dipalmitate was determined spectrophotometrically at 266 nm after solubilization in methanol. Typically, more than 95% of the prodrug was incorporated in the liposomes. The liposome dispersions were stored under nitrogen at 4°C, and were used within two weeks of preparation.

Lytic effects of liposomal FUdR-dipalmitate on red blood cells

Fresh blood from healthy human volunteers was collected in heparinized tubes. After centrifugation the cells were washed three times with phosphate buffered saline (PBS, pH = 7.4). The cells were diluted with PBS to a hematocrit of 10%. Samples of this erythrocyte suspension (50 μl) were mixed with different concentrations of test compounds (liposomal FUdR-dipalmitate or FUdR-dipalmitate in ethanol) and the volumes adjusted to 1 ml with PBS. The mixtures were incubated for 30 min at 37 °C. After incubation, the suspensions were centrifuged for 3 min in an Eppendorf centrifuge (at 12,000 x g) to remove the cells. The supernatants were centrifuged once more to remove the liposomes (30 min 40,000 rpm). The absorbance of the supernatant at 541 nm was determined. The extent of hemolysis was then calculated, using the absorbance value of a 0.5% erythrocyte suspension in water as reference for 100% hemolysis.

Assay of hemopoietic colony forming cells in bone marrow and spleen

Female Balb/c mice (8-10 weeks) were injected for 6 consecutive days (intravenously day 1-3 (tail vein) and intraperitoneally day 4-6) with FUdR (600 μmol/kg), with FUdR-dipalmitate in DSPC/DPPG/CHOL liposomes (2 μmol/kg FUdR-dipalmitate, 20 μmol of total lipid/kg), or with empty liposomes (20 μmol of total lipid/kg). The injected volume was 200 μl. One day after the last injection the mice were anesthetized with diethyl ether and blood was collected from the retro-orbital sinus in heparinized tubes. The hematocrit of the blood samples was determined. The number of nucleated cells was determined in a Coulter counter (Coulter Electronics LTD, Dunstable Beds, England). To identify the presence of bactria, blood samples were serially diluted in 10-fold dilution steps in Brain Heart Infusion broth (Difco). After overnight incubation (37 °C), and dilutions were subcultured on MacConkey agar (Oxoid) to identify the presence of aerobic gram negative bacteria.
After blood collection, the mice were killed by cervical dislocation and the spleen and one femur were excised under sterile conditions. Bone marrow cell suspensions were obtained by flushing the femur with 1 ml α-medium (Gibco) plus 10 mM HEPES, pH 7.2. Single cell suspensions were made by repeated flushing through a 25-gauge needle. Nucleated cell count was determined in a Coulter Counter and bone marrow cell number was calculated, assuming that one femur contains 6% of total bone marrow.
Spleens were gently pressed through a stainless steel sieve 100 mesh) and suspended in α-medium through 20- and 25-gauge needles, respectively. The femoral and splenic nucleated cells were counted in a Coulter counter. The assay of colony forming units of granulocyte/macrophage lineage (CFU-GM), burst forming units of erythrocyte lineage (BFU-E) and colony forming units of erythrocyte lineage (CFU-E) was performed in a methylcellulose medium (17) with the appropriate growth factors as described earlier (18).
Cytospin preparations (Shandon centrifuge, Zeist, The Netherlands) of femoral and splenic cell suspensions were made for morphologic differentiation of the cells after staining (May Grünwald Giemsa).

Histopathology of liver, spleen and ileum

Female NMRI mice (8-10 weeks) were injected with FUdR, FUdR-dipalmitate in liposomes or empty liposomes as described above. During therapy the body weight of the mice was monitored.

One day after the last injection the mice were killed by cervical dislocation. Liver, spleen and part of the ileum were excised, weighed and fixed in formalin (4 %v/v, in phosphate buffered saline 0.67 mM). Microscopic preparations of the organs were made and stained with hematoxilin/eosine.

Statistical analysis

Student's t-test was used for statistical analysis.

RESULTS

Lytic effects of FUdR-dipalmitate on erythrocytes.

The first cells coming into contact with drugs upon intravenous injection are the erythrocytes. To investigate the possibility of direct cell lysis leading to anemia, the prodrug was incubated with erythrocytes for 30 min at 37°C and cell lysis was measured. The drug was found to induce hemolysis, but only when added to the cells in ethanol. Liposomes, on the other hand, protected the erythrocytes against the toxicity of FUdR-dipalmitate: only 0.4 % lysis was registered when the drug was incorporated in liposomes (DSPC/DPPG/CHOL) whereas 52.0 % lysis was observed when the drug was dissolved in ethanol (at a concentration of 0.5 mM). It is important to note that ethanol and empty liposomes were not toxic at the concentrations used, as can be seen from table I.
The highest plasma concentration of liposomal FUdR-dipalmitate that will be obtained in vivo, after injection of 2 μmol/kg, is about 0.04 - 0.06 mM (assuming instantaneous mixing of liposomes and plasma, and assuming a plasma volume of 0.7 - 1 ml and a body weight of 20 g per mouse). This implies that the drug concentration obtained in vivo is lower than the one used in the lysis experiments, and thus direct toxicity of FUdR-dipalmitate to cells is considered to be negligible in the overall toxicity. FUdR, which was not tested in this experiment, has been found by others to induce 3% hemolysis at a concentration of 350 mM (19). Based upon the assumptions outlined above, we typically obtained an initial concentration of 6 - 8 mM in vivo upon injection of 600 μmol/kg.

Macroscopic symptoms of toxicity

The condition of mice treated with liposomal FUdR-dipalmitate deteriorated between the third and fourth day of the treatment. This was seen as a decrease in body weight due to reduced food intake and diarrhea (in some mice). Furthermore, the mice showed symptoms of dehydration, felt cold upon touching, were inactive and quivering and their fur looked unhealthy. Severeness of the illness increased upon further treatment, reaching its maximum between day seven (one day after the last treatment) and day nine. After this

crisis, the animals recovered surprisingly fast. In FUdR-treated mice, the same signs of toxicity were observed, only less severe.

In order to further examine toxicity at the organ level, mice were sacrificed one day after the last drug injection (when toxicity was maximal). At obduction the following signs of toxicity were observed:

- Liver and spleen were reduced in size (table II); the spleen was almost transparent. Livers of mice treated with higher drug doses contained cirrhotic spots and reddish lobule-rims.
- The thymus was exceptionally small.
- An accumulation of gas was found in the small intestine. Earlier, we found that the gastro-intestinal tract of tumor-bearing mice that died of drug toxicity was inflamed and hemorrhaged (4).

Table I.
Toxicity of FUdR-dipalmitate to erythrocytes

Preparation	concentration total lipid (mM)	concentration FUdR-dipalmitate (mM)	% hemolysis
DSPC/DPPG/CHOL	5	-	1.2
DSPC/DPPG/CHOL	5	0.5	0.4
	1	0.1	0.7
ethanol*	-	0.5	52.0
	-	0.1	7.5
	-	0.05	6.5
	-	0.01	4.1
ethanol*	-	-	1.2

Empty liposomes or FUdR-dipalmitate in liposomes or ethanol were incubated at 37 °C with a 0.5% erythrocyte suspension. After 30 min, the cells and the liposomes were removed by centrifugation and the absorbance of the supernatant at 541 nm was read. The percentage hemolysis was calculated, taking the absorbance of 0.5% erythrocyte suspensions in water as the 100% hemolysis value.

* the highest ethanol concentration was 4.5% (v/v).

Histopathology of liver, spleen and ileum

Histological examination of liver, spleen and ileum showed the toxicologic damage to these organs at a microscopic level:
Liver. The morphological structure of the liver, the sinusoids, the portal triads and the parenchymal cells appeared normal. The only difference with control livers was the absence of mitoses in the livers of animals treated with FUdR or FUdR-dipalmitate. Mitoses in control livers were seen in 2% of the hepatocytes.
Spleen. The spleens of treated animals were affected. The cell numbers were decreased in all areas (the red and white pulp as well as the marginal zone between the red and white pulp). This was particularly apparent at the peripheral side. The capsule around the spleen was wrinkled, in contrast to that of control mice.
Ileum. Figures 1a - 1f show the microscopic appearance of the ileum of the control and liposomal FUdR-dipalmitate treated mice. The structure of the villi and the crypts was disordered when the mice were treated with FUdR-dipalmitate. Furthermore, granulocytes can be seen around the crypts, whereas they are absent in the small intestines of control animals. Also, in the ileum of treated animals clumps of dead cells are observed. The number of columnar epithelium cells is decreased after liposomal FUdR-dipalmitate treatment and nuclei are enlarged and vesiculated, containing very prominent nucleoli.

The pathological findings as described above for liposomal FUdR-dipalmitate (2 μmol/kg) were also observed in mice treated with FUdR (600 μmol/kg). However, the structural damage caused by FUdR was generally less severe (not shown). The same histological findings were observed when 5FU was injected (110 μmol/kg).

Table II:
Effects on body, liver and spleen weight caused by treatment with FUdR or liposomal FUdR-dipalmitate

Treatment	BWD (%)*	liver (g)	spleen (g)
buffer	+ 10	1.65	0.14
empty liposomes	+ 4	1.44	0.16
FUdR (n=3)	- 19	1.18 ± 0.07	0.068 ± 0.003
Lip-FUdR-dipalmitate (n=3)	- 18	0.95 ± 0.15	0.069 ± 0.019

Mice were treated for 6 consecutive days with FUdR (600 μmol/kg) or liposomal FUdR-dipalmitate (2 μmol/kg). One day after the last injection the body weight of the mice was measured and expressed as the percentage of the weight before start of the treatment. Liver and spleen were excised and weighed after fixation in 4% formaldehyde solution.
* BWD = body weight difference.

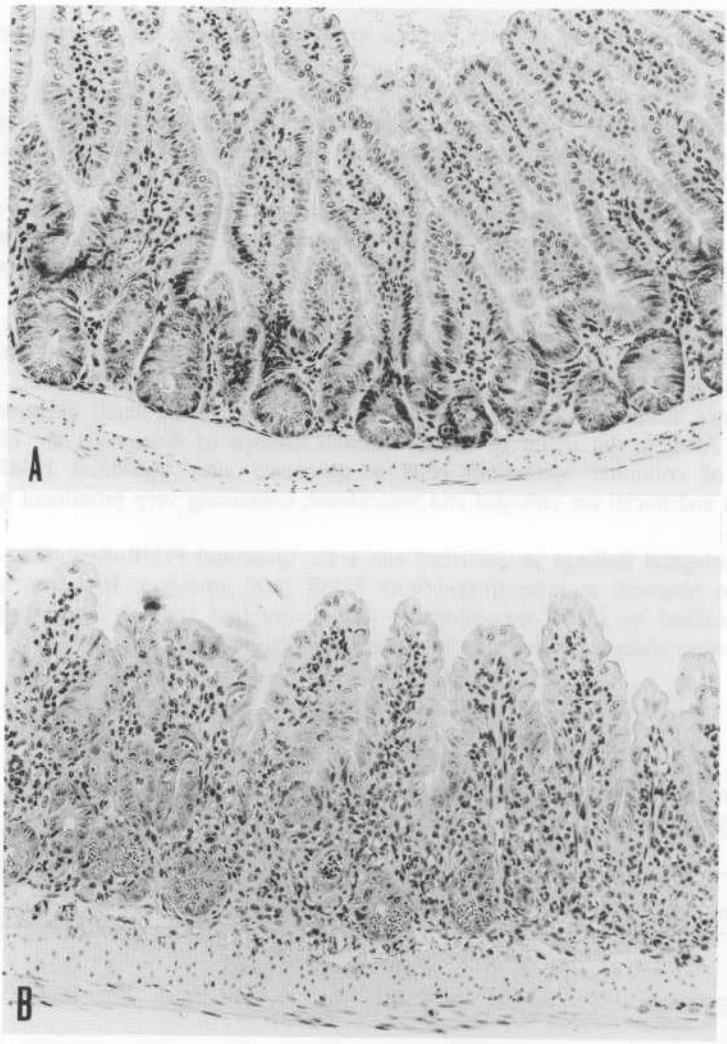
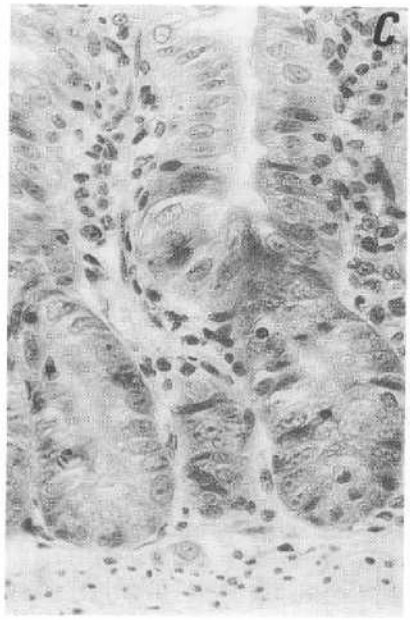


Fig. 1
Observations of mouse ileum at low power magnification shows regularly arranged villi and crypts with highly ordered columnar epithelial cells in untreated (A), and damaged villi and crypts in liposomal FUdR-dipalmitate treated mouse (B) (160 x). At a higher magnification, crypts (untreated (C), liposomal FUdR-dipalmitate (D)) and villi (untreated (E), liposomal FUdR-dipalmitate (F)) show these differences more clearly: granulocytes (⬇) and cell debris (⇊)(D), loss of columnar epithelial cells, as well as enlarged nuclei with prominent nucleoli (↟) can be seen after treatment (F) (410 x).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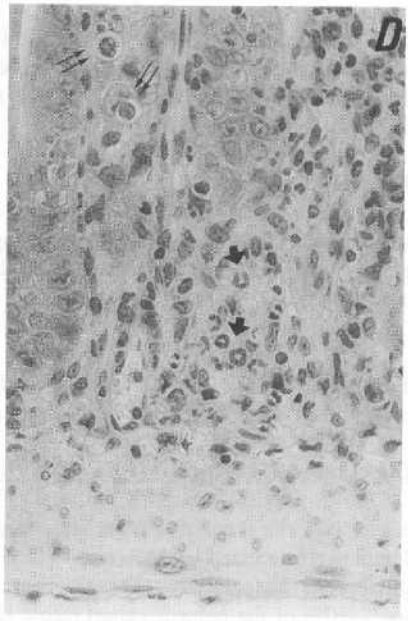
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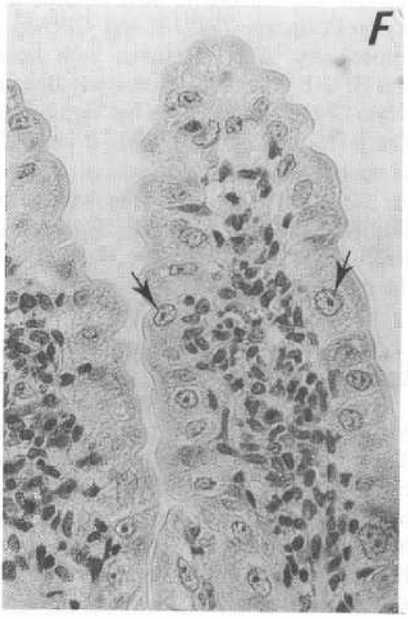
F

Evaluation of blood parameters (hematocrit, WBC and sterility)

After treatment of mice for 6 consecutive days with FUdR (600 μmol/kg) the hematocrit and total white blood cell count were decreased. Liposomal FUdR-dipalmitate (2 μmol/kg) decreased the white blood cell count to the same level as FUdR but did not cause a decrease in the hematocrit (table III).

To assess the possibility of bacterial infiltration from severely compromised intestinal tissue, the sterility of blood samples of treated mice was tested by incubating blood samples in broth and MacConkey's agar. No bacterial growth was detected in either growth medium supplemented with blood from FUdR or liposomal FUdR-dipalmitate treated mice.

Toxicity of FUdR and liposomal FUdR-dipalmitate to granulopoietic or erythropoietic progenitor and precursor cells in bone marrow and spleen

Effect on progenitor cells (CFU-GM, BFU-E and CFU-E)

Both FUdR and liposomal FUdR-dipalmitate decreased the total number of nucleated cells recovered from bone marrow and spleen. The nucleated cell numbers in the femur were reduced to 28% and 73% of the control value by FUdR and liposomal FUdR-dipalmitate respectivily. The decrease in spleen cells was the same for both drugs (60%-70% of the control value) (table III).
The progenitor cell population of the granulocyte/monocyte lineage (CFU-GM) in bone marrow was reduced to 30% of control levels after treatment with FUdR. The cells of the erythroid lineage (BFU-E and CFU-E) were decreased to 20 and 40% of control values, respectively. After treatment with liposomal FUdR-dipalmitate, bone marrow CFU-GM and BFU-E were only moderately decreased to 74 and 90% of control values, respectively, while CFU-E counts were not significantly different from controls.
The levels of CFU-GM, BFU-E and CFU-E in the spleen were all decreased to 40 - 50% of control values after treatment with liposomal FUdR-dipalmitate. The subpopulations of the different progenitor cells were reduced more after treatment with FUdR than after FUdR-dipalmitate treatment. Furthermore, FUdR and FUdR-dipalmitate both suppressed BFU-GM and BFU-E more than CFU-E, while the suppressing effects of both drugs on the different progenitor cells were more rigorous in the spleen than in the bone marrow.

Effect on morphologically recognizable precursor cells

In order to determine the effect of FUdR and liposomal FUdR-dipalmitate on morphologically distinguishable erythroid, granuloid and lymphoid precursors, we determined the numbers of these cells in bone marrow and spleen (table IV). FUdR induced a significant reduction of the cells of the erythroid and granuloid cell lineages, both in bone marrow and in spleen. The number of lymphoid precursor cells after FUdR-treatment was 70% - 80% of control values in bone marrow and in spleen. Liposomal FUdR-dipalmitate decreased the lymphoid precursor cells in the spleen considerably (to 66% of control values), whereas the lymphoid precursor cells in the bone marrow were not

affected. On the whole, the effect of FUdR-dipalmitate on precursor cells equaled the effect of FUdR on these cells for the spleen, but was less severe for bone marrow.
Morphological aberrations were seen particularly in bone marrow granulopoietic cells after treatment with FUdR: abnormal segmentations and megaloblastic appearance with extreme vacuolization were observed (fig. 2a-c). Furthermore, there were relatively few late erythroid precursor cells, and thus a shift to early precursors was observed (not shown). These findings were the same after treatment with FUdR-dipalmitate but also in this case the toxicological aberrations were less extensive. In spleen cells, only minor morphological aberration were observed.

Table III.
Effect of free FUdR and liposomal FUdR-dipalmitate on murine bone marrow and spleen hemopoietic cells

	BLOOD		BONE MARROW			
TREATMENT	Hct	WBC (10^6/ml)	TNC (10^6)	CFU-GM (10^3)	BFU-E (10^3)	CFU-E (10^3)
control	47.3 ± 0.3	6.1 ± 0.5	279 ± 29	85 ± 9	102 ± 9	816 ± 119
FUdR	43.7 ± 1.0	2.8 ± 0.8	78 ± 3	26 ± 7	20 ± 14	328 ± 153
Lip-FUdR-dipalmitate	47.3 ± 1.2[a]	3.3 ± 1.2[b]	204 ± 20	69 ± 10[c]	92 ± 29[c]	913 ± 209[a]

	SPLEEN			
TREATMENT	TNC (10^6)	CFU-GM (10^3)	BFU-E (10^3)	CFU-E (10^3)
control	183 ± 32	2.2 ± 0.4	2.3 ± 0.5	33.0 ± 1.0
FUdR	126 ± 27	0.2 ± 0.1	0.1 ± 0.1	8.0 ± 2.9
Lip-FUdR-dipalmitate	109 ± 8[b]	1.0 ± 0.3	1.0 ± 0.2	18.0 ± 5.7[c]

Mice were injected 6 times with FUdR (600 μmol/kg) or liposomal FUdR-dipalmitate (2 μmol/kg). One day after the last injection the mice were killed and the spleen and femur removed. A suspension of spleen and femur marrow cells was prepared in which the total nucleated cell content (TNC) was determined. The cells were grown under conditions described in the materials and methods section. Total bone marrow cells were calculated, assuming that one femur contains 6% of the total bone marrow. The results given are of a typical experiment; each group consisted of three mice.
a: values are not significantly different from control
b: values are not significantly different from FUdR
c: $p < 0.05$ (difference from control); for all other values $p < 0.02$.

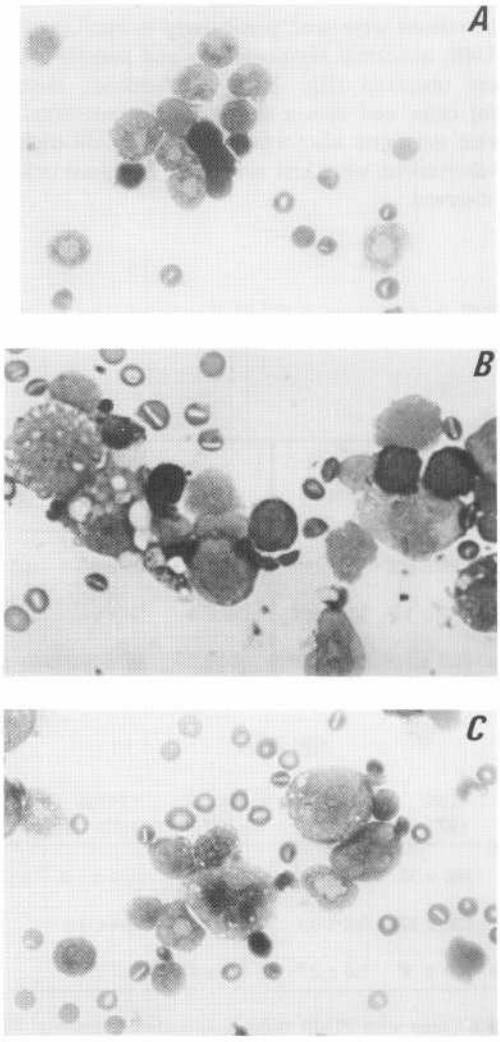

Fig. 2
High power magnification of bone marrow cells of untreated mice (A) and after FUdR treatment (B and C). Note the enlarged, vesiculated cells of the latter (1024 x).

Table IV:
Morphological classification of mouse bone marrow and spleen cells in erythroid, granuloid and lymphoid cells.

BONE MARROW

TREATMENT	TNC	E	G	L	U
none	279	77	150	44	10
FUdR	78 (28)*	14 (18)	34 (23)	31 (69)	2
Lip-FUdR-dipalmitate	204 (73)	46 (60)	100 (68)	51 (115)	5

SPLEEN

TREATMENT	TNC	E	G	L	U
none	183	20.7	8.7	151	2.8
FUdR	126 (69)*	4.8 (23)	0.9 (10)	119 (79)	1.6
Lip-FUdR-dipalmitate	109 (60)	6.9 (33)	1.4 (16)	100 (66)	0.4

After treatment for 6 consecutive days with FUdR (600 μmol/kg) or liposomal FUdR-dipalmitate (2 μmol/kg) mice were killed. From each animal spleen and bone marrow (femur) were recovered and homogenized as described in materials and methods. The total number of nucleated cells (TNC) in the cell suspensions was determined. Two hundred cells on each slide were classified as either erythroid (E), granuloid (G), lymphoid (L) or unidentified (U). The magnitude of the original cell population was calculated from TNC, after assessing the relative number of cells in percentage in each of the different groups. The numbers in parentheses represent the percentage of the different cell populations from treated mice with respect to control mice.
The numbers given represent the number of cells x 10^6. Each value represents the mean of three independent observations.
* percentage of control values.

DISCUSSION

In search of a new administration form of FUdR we tested a prodrug formulation i.e. FUdR-dipalmitate incorporated in liposomes. During studies, we and others found that the therapeutic dose of liposomal FUdR-dipalmitate in mice given repeated intravenous or intraperitonal injections, was 60 - 300 times lower than that of the parent compound (1-4). At this low drug dose, liposomal FUdR-dipalmitate induced toxicity symptoms in the mice, which were more severe than found after bolus injections of high doses of FUdR. The two administration forms differ in the duration at which they expose tissues to active drug in vivo: FUdR given as a bolus injection is rapidly catabolized to the less active 5-fluorouracil (5FU) and inactive catabolites (5,6), while liposomal FUdR-dipalmitate is mainly deposited in macrophages of liver and spleen, where liposomes and prodrug are degraded intralysosomally, causing a sustained release of FUdR from the cells (9).

The principal goal of this study was to examine whether the toxic mechanisms of FUdR and FUdR-dipalmitate differ with respect to the type of affected tissues and/or to the type of cellular damage. Therefore, at the time of maximal toxicity (after 6 days of treatment with 2 μmol/kg liposomal FUdR-dipalmitate or 600 μmol/kg FUdR), damage to bone marrow, spleen, liver and ileum of mice were examined.

Hemopoietic system. The clinical manifestations of hemopoietic toxicity of FUdR are similar to those of 5FU, the first catabolic degradation product of FUdR in vivo. This toxicity comprises: leukopenia, thrombocytopenia and anemia (11,20). We also observed leukopenia and anemia after repeated administration of FUdR to mice. On the other hand, administration of liposomal FUdR-dipalmitate led only to a decrease in white blood cell count whithout affecting the hematocrit. This does not necessarily indicate that FUdR-dipalmitate has no effect on red blood cell development. A decrease of red blood cells might have remained unnoticed due to the dehydrating effect of the drug on the mice, which can lead to a reduction in plasma volume (leading to an increased hematocrit).

The effect of 5FU on the hemopoietic system and especially on the individual subsets of hemopoietic cells has been thoroughly studied (21-27), in contrast to such effects of FUdR. The toxicity of 5FU is acute but reversible after administration of a non-lethal dose of the drug. 5FU reduces total cell numbers in spleen and bone marrow, including a decrease in progenitors (BFU-E, CFU-E and CFU-GM) and precursors. The primitive pluripotent hemopoietic stem cell is not damaged and is able to repopulate the hemopoietic compartment (28-31). As could have been expected, considering the rapid conversion of FUdR to 5FU in vivo, the toxic effects of 5FU resemble those of FUdR also at the cellular level. In our experiments we found a reduction in the total nucleated bone marrow and spleen cell count, and a decrease in progenitor and precursor cells of the erythroid and granuloid/macrophage lineage after repeated administration of FUdR to mice. The suppressing effect of liposomal FUdR-dipalmitate was less than the effect of FUdR, both in spleen and in bone marrow.

Apart from the toxic effects on the hemopoietic progenitor and precursor cells, the immune system was also impaired. This was measured as a reduction of thymus and spleen volume and of the number of lymphoid precursor cells in the spleen and of lymphocytes in the blood.

Liver and ileum. Histologically, no difference was found between FUdR and FUdR-dipalmitate with respect to the nature of the toxic effects on liver, spleen and ileum. For the ileum, however, quantitative differences in the severyness of the observed effects, with FUdR being less toxic than FUdR-dipalmitate in the applied dose (FUdR 600 μmol/kg, FUdR-dipalmitate 2 μmol/kg). The liver weight reduction was considerable in both cases and was higher than the reduction in body weight, but no cellular debris could be demonstrated. Also, the portal triad, which is damaged after hepatic arterial infusion of FUdR to humans (12-15), was not affected. The only abnormality was the absence of mitoses in livers of treated mice, while these were common in the livers of control animals. These findings were similar to those obtained after portal vein infusion of FUdR in rats, where a considerable reduction in liver mass was found (-30%) which was said to be caused by loss of liver parenchymal cells. Due to this loss, a decrease of liver enzymes (SGOT, SGPT and AP) in the blood was found (32). We also found a decrease in liver enzymes in rat plasma after treatment with liposomal FUdR-dipalmitate (10 μmol/kg/day) (unpublished data).

We did not observe abnormal nucleoli in liver cells, as was reported earlier after injection of a high dose of 5FU (33, 34). However, we observed nucleoli of abnormal size in enterocytes of the ileum of treated mice. The finding of enlarged nucleoli is not unusual after treatment of cells with 5FU (35-37). This is generally explained as an effect of the fluoropyrimidines at the RNA-level: 5FU is converted to 5-fluorouridine-triphosphate (FUTP) which can be incorporated in all species of RNA. Its most pronounced effect is the inhibition of the maturation of ribosomal RNA, coupled to the storage of higher molecular weight pre-ribosomal RNA-segments. The storage of such segments may lead to the enlargement of the nucleolus (34,38).

It is of interest to note that we found enlarged nucleoli in the enterocytes of ileum of mice treated with liposomal FUdR-dipalmitate. If this is to be explained as an RNA-effect, FUdR has to be liberated from the liposomal prodrug, converted to 5FU and then anabolized to the active nucleotide FUTP. However, to induce the effect on the nucleoli, high doses of 5FU are necessary, much higher than we used for liposomal FUdR-dipalmitate. Thus, excluding rapid conversion of the lipophilic prodrug to FUTP, we have to assume that it is unlikely that the enlargement of the nucleoli in the nucleus of the enterocytes to be caused by an effect at the RNA-level.

Apart from the enlarged nucleoli, we found other abnormalities in the ileum, common to damage induced by 5FU or other cytotoxic agents (39-41), i.e. inflammation, granulocyte infiltration, the occurrence of cell debris and the loss of columnar epithelium cells. Damage to the intestinal mucosa can decrease the physical condition of the mice due to diarrhea, dehydration, and malabsorption of nutrients. Subsequently, bacterial infiltration and sepsis can develop and, combined with an impaired immune system, can ultimately lead to death.

In summary, FUdR can be repeatedly given to mice in a dose upto 600 μmol/kg, whereas the maximal tolerated dose of liposomal FUdR-dipalmitate is 2-10 μmol/kg/day. Given at these doses both drugs depress bone marrow and spleen cell proliferation, reduce liver mass and induce abnormalities in the ileum (cell debris, loss of columnar epithelial cells, enlarged nuclei with prominant nucleoli in these cells, and granulocyte infiltration). The toxicity of FUdR to the hemopoietic tissue is more severe than that of liposomal

FUdR-dipalmitate. In contrast, toxicity to the ileum is more severe for liposomal FUdR-dipalmitate than for the parent drug. Thus, a shift from hemopoietic to gastro-intestinal toxicity is seen when the residence time of FUdR in the body is prolonged by the liposomal formulation. This has also been observed in the clinical situation (10-13) when the drug is given as an intravenous infusion instead of a bolus injection.

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